Heparin-Binding Proteins on Bull, Boar, Stallion, and Human Spermatozoa

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ABSTRACT

The seminal plasma from many mammalian species contains heparin-binding proteins which bind to the sperm surface at ejaculation and appear to mediate sperm capacitation modulated by glycosaminoglycans (GAG) present in the reproductive tract of the female. The molecular mechanisms underlying this process is only poorly understood, however. Furthermore, seminal plasma heparin-binding proteins have been structurally characterized in only few mammalian species. Here, we summarize our present knowledge on sperm surface-associated heparin-binding proteins in four different mammals: boar, bull, stallion and man. The major conclusion of this comparative study is that the major heparin-binding proteins from these species belong to only three different protein families. The relative abundance of proteins of different families on spermatozoa from the different species, together with structural diversity between proteins of the same family in different species, and species-specific topographical localization of homologous heparin-binding proteins on spermatozoa, may contribute to the specific spermatozoon phenotype, which in turn may modulate species-specific effects of GAGs on sperm capacitation.

RÉSUMÉ

Les protéines liant l'héparine dans les spermatozoïdes du Taureau, du Verrat, du Cheval et de l'Homme

Le plasma séminal de nombreuses espèces de mammifères contient des protéines liant l'héparine qui se lient à la surface des spermatozoïdes à l'éjaculation et semblent être les médiateurs de la capacitation des spermatozoïdes, qui est modulée par les glycoaminoglycanes (GAG) présents dans le tractus génital de la femelle. Toutefois, les mécanismes moléculaires sous-jacents à ce processus sont mal connus. De plus, la structure des protéines du plasma séminal liant l'héparine a été caractérisée seulement dans quelques espèces animales. Nous résumons ici l'état actuel des connaissances sur les protéines liées à l'héparine associées à la surface des spermatozoïdes chez quatre mammifères: Verrat, Taureau, Cheval et Homme. La conclusion principale de cette étude comparative est que les protéines majeures liant l'héparine de ces espèces appartiennent à seulement trois familles différentes de protéines. L'abondance relative des protéines de différentes familles sur les spermatozoïdes des différentes espèces, ainsi que la diversité structurale entre les protéines d'une même famille dans les différentes espèces, et que la localisation topographique spécifique à l'espèce des protéines homologues liant l'héparine sur les spermatozoïdes, peuvent contribuer au phénotype spécifique du spermatozoïde, qui ensuite module les effets spécifiques des glycoaminoglycanes sur la capacitation des spermatozoïdes.

CALVETE, J. J., SANZ, L., REINERT, M., DOSTÁLOVÁ, Z. & TÖPFER-PETERSEN, E., 1995. — Heparin-binding proteins on bull, boar, stallion, and human spermatozoa. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 515-524. Paris ISBN : 2-85653-225-X.

Following the differentiation of the haploid spermatozoon, the cell is released from the epithelium of the seminiferous tubules passing from the testis to the epididymis. Mammalian spermatozoa are highly differentiated by the time they leave the testis. Nonetheless, at this stage, they do not have the ability to fertilize eggs. Spermatozoa gain this capability while passing through the epididymis (epididymal maturation) and/or after residing in the female tract for some period of time (capacitation) (reviewed in [35]). Though our knowledge of the molecular basis of these processes is still in its infancy, one of the most prominent, and best documented, physiological changes in the spermatozoa is a continous remodelling of their plasma membrane components as spermatozoa travel through the various microenvironments within the male and the female genital tracts [17, 35]. Both membrane-integrated and surface-adsorbed components (lipids or proteins) either change their location in or on the plasma membrane, are altered, masked, or replaced as a response to the constantly changing osmolarity and chemical composition of the millieu surrounding the sperm. In particular, at the time of ejaculation, spermatozoa from the distal (cauda) epididymis are mixed with secretions of the male accessory sexual glands. Recent evidence suggests that components of seminal fluid upon attaching to the sperm surface may regulate important sperm functions: some (glyco)proteins stabilize the plasma membrane and may prevent premature acrosome reactions, and others are believed to mediate interactions between spermatozoa and the zona pellucida. Here, we will restrict the discussion to the structure and proposed biological role of heparin-binding proteins of the seminal plasma of different mammals: boar, bull, stallion and man. Interestingly, the seminal plasma heparin-binding proteins of these four species belong to only three different protein families, which include both capacitation factors and carbohydrate-recognition (putative zona pellucida-binding) molecules. Differences between species in the relative abundance and topography of proteins from different families on the sperm surface, may contribute to the species-specific physiology of mammalian spermatozoa.

RESULTS AND DISCUSSION

Heparin-like GAGs and heparin-binding proteins in fertilization

Proteoglycans with heparin- and chondroitin sulphate-like glycosaminoglycan side chains are secreted by the epithelium of the female reproductive tract, particularly at high concentration during the follicular phase of the estrous cycle, and have been shown to specifically invoke sperm capacitation in a number of mammalian species, as measured by the onset of agonist-inducible acrosome reactions [32]. Thus, *in vitro* incubation of epididymal sperm from such different mammalian species as bovine, hamster, human, rabbit, or equine, with glycosaminoglycans significantly accelerated the development of the sperm capacitated state as judged by their increased ability to fertilize homologous eggs. In addition, since exposure of bovine epididymal spermatozoa to seminal plasma from the same species enhanced their ability to undergo the acrosome reaction in the presence of heparin [25], it follows that the effects of heparin-like GAGs appear to be mediated by seminal plasma heparin-binding proteins which coat the sperm surface at ejaculation.

Seminal plasma heparin-binding proteins on bovine spermatozoa

Comparison of the heparin-binding protein composition of epididymal and ejaculated spermatozoa showed the presence of a 30 kDa and several 15-17 kDa proteins in the ejaculated spermatozoa [10]. The same group of proteins were adsorbed to the sperm surface following exposure of epididymal sperm to seminal plasma [24]. The 30 kDa protein has not been structurally characterized. The primary structures of the 15-17 kDa proteins, designated BSP-A_1, BSP-A_2 and BSP-A_3, have been reported [16, 30]. BSP-A_1 and BSP-A_2 have the same polypeptide backbone but the former contains a single O-glycosylated trisaccharide (NeuNAc α(2-6)-Gal ß(1-3)-GalNAc-) attached at threonine-11 [5], and together they are also known as Major

Protein (MP) or PDC-109 (Fig. 1A). Structurally, BSP-$A_{1/2}$ and BSP-A_3 are made up of two tandemly-arraged homologous modules of around 40 residues that share the consensus sequence of the collagen- and heparin-binding type-II modules of fibronectin [30] (Fig. 1A). Each of these repeats contains the amino acid residues which have been identified by alanine scanning mutagenesis to be critical for collagen binding [2]. In addition, the isolated B-domain of PDC-109 retained affinity for immobilized collagen [2]. Recently, a refined H^1-NMR-solution structure of the 45 -residue PDC-109 domain B has been reported [12]. However, the folding pattern of the whole molecule and the heparin-binding site(s) remain to be established.

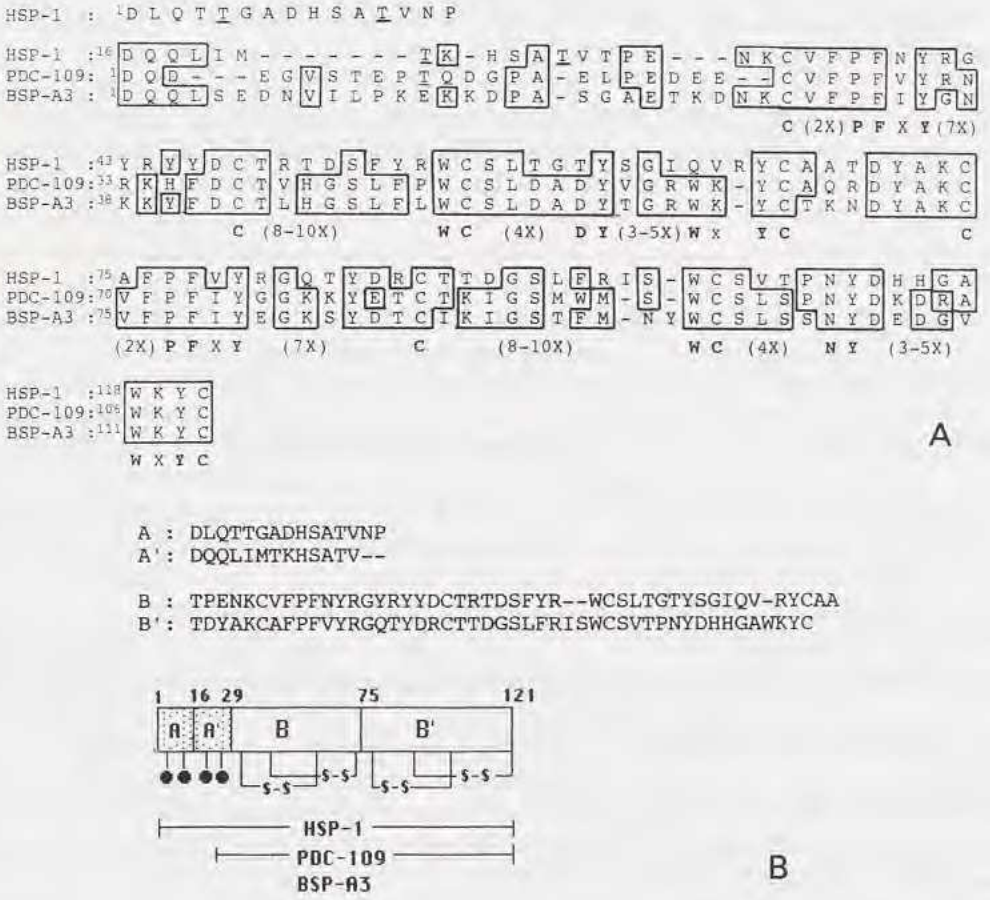

Fig. 1. — **A**: Comparison of the primary structure of the major heparin-binding proteins from stallion (HSP-1) and bovine (PDC-109 and BSP-A3) seminal plasma. Identical residues in at least two proteins are shown in boxes. The consensus amino acids of the fibronectin type II module and the spacing between them are shown below the sequence alignment. T, O-glycosylated threonine residues. **B:** Amino acid sequence alignment of the A- and B-type internal repeats of stallion heparin-binding protein HSP-1, and a scheme of the domain arrangement in HSP-1 and in bovine PDC-109 and BSP-A3. –•, indicates the relative positions of O-glycosylation sites; S-S, disulphide bridge.

Proteins cross-reacting with monospecific antibodies against either BSP-$A_{1/2}$, BSP-A_3, or BSP-30 kDa have been detected in the seminal fluids of human, porcine, hamster, mouse, and rat [18]. However, only the gene expression of bovine BSP-$A_{1/2}$ has been studied [29]. Southern

blot analysis of genomic DNA indicated that BSP-A_1/A_2 is coded for by a single gene per haploid bovine genome [29]. The protein is secreted by the seminal vesicle epithelium at a concentration of 16-25 mg/ml [5, 29].

The topographical localization, the nature of the sperm surface acceptor molecules of PDC-109, and its physiological role are controversial. Thus, AUMÜLLER *et al.* [1] reported that PDC-109 binds preferentially to the middle piece and neck region of bovine spermatozoa and identified by immunoblotting analysis a 65-67 kDa protein duplet as a PDC-109 acceptor site on epididymal spermatozoa. These authors postulated that binding of PDC-109 to its acceptor site might be regarded as a physiological event related to the onset of hyperactivated sperm motility [1]. On the other hand, Manjunath et al. have shown that isolated PDC-109 possesses the ability of binding a number of ligands, such as apolipoprotein A-I (apoA-I) and apoA-I associated high-density lipoproteins, different types of collagen (I, II, IV, and V), fibrinogen, heparin, calmodulin, phospholipase A_2 (PLA_2), and phosphorylcholine-containing lipids [23]. Using immunofluorescence, MANJUNATH *et al.* have reported that BSP-$A_{1/2}$ is located on the entire sperm head surface, "although the intensity was noticeably stronger at the midpiece region" [22]. These authors have proposed that BSP-$A_{1/2}$ may play an important role in sperm lipid modification that occurs during capacitation and the acrosome reaction. In their mechanistic theory, binding of BSP-$A_{1/2}$ to choline phospholipids on the sperm surface may block PLA_2 from acting on these phospholipids and prevent sperm from undergoing a premature acrosome reaction. In addition, acting as decapacitation factors sequestering cholesterol and choline phospholipid, BSP-$A_{1/2}$ may alter the fluidity and permeability of the spermatozoal membrane and allow calcium to enter for activation of PLA_2, which would then produce lysophospholipids that are known to destabilize membranes and trigger membrane fusion (acrosome reaction).

In addition to the proposed role in membrane lipid remodelling events, MANJUNATH *et al.* [22] have reported a specific interaction of BSP-$A_{1/2}$, BSP-A_3, and BSP-30 kDa with insulin-like growth factor-II (IGF-II), and hypothesized that BSP-proteins bound to the cell surface could modulate the IGF-II action by serving as carriers as well as cell surface binding site for the hormone.

Our own data showing that, upon mixing of spermatozoa with seminal plasma at ejaculation, 9 million molecules of PDC-109 on average become coated to the sperm surface, and that this figure decreases only to 8 million molecules per spermatozoon after incubation for 24 h in capacitation medium at 39° C [5], do not support a role for BSP-$A_{1/2}$ as a decapacitation factor. Furthermore, BSP-$A_{1/2}$ eluted from a Sephadex G-200 gel filtration column as aggregated molecules with an apparent molecular mass of 60-120 kDa [22], indicating that in addition to binding to 65-67 kDa acceptor protein(s) and choline phospholipids, BSP-A1/2 becomes coated to the sperm surface as a multimer. How the aggregation state of BSP-A1/2 affects the various ligand-binding activities reported in *in vitro* systems deserves further investigation. Clearly, much work is needed to establish the actual biological function(s) of, and the mechanisms used by, the different members of the bovine seminal plasma (BSP) protein family on sperm physiology.

Another abundant heparin-binding component of bovine seminal plasma (~7 mg/ml) is acidic seminal fluid protein (aSFP) [15]. Deduction of the primary structure of aSFP from a complete cDNA clone [34] showed that aSFP shares 43% sequence identity with porcine spermadhesins (see below) (Fig. 2). On average, 6 x 10^6 molecules of aSFP bind to a narrow region on the apical part of the acrosome of an ejaculated spermatozoon, but this amount decreases to undetectable levels in capacitated sperm [14]. This indicates that aSFP might have a role as a decapacitation factor, and shows that proteins from the same family (spermadhesin) play different functions in the fertilization process of different mammalian species, i.e. in bull and boar (see next section).

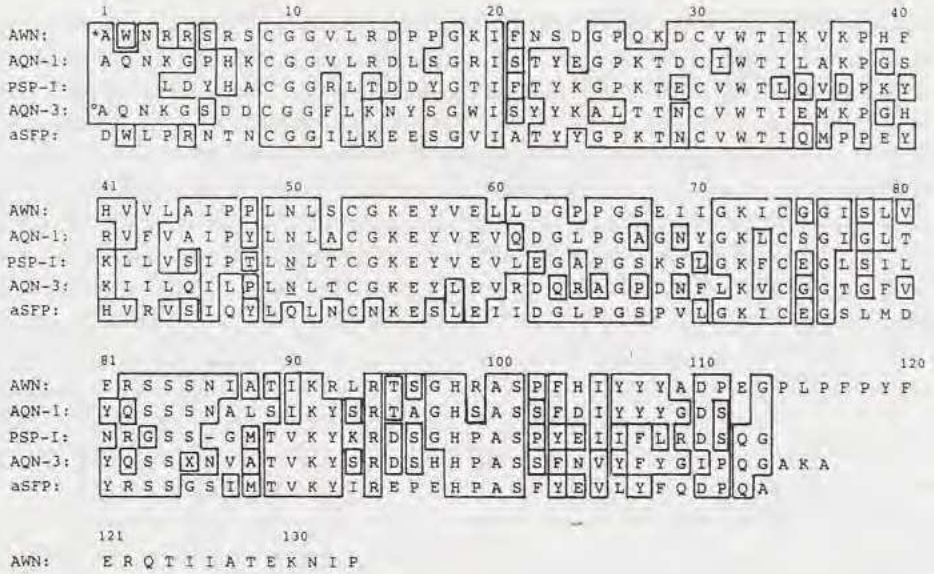

FIG. 2. — Alignment of the amino acid sequence of boar spermadhesins AWN, AQN-1, PSP-I, and AQN-3, and the bovine seminal plasma polypeptide aSFP. Identical residues within at least two of these proteins are shown in boxes. *, this residue is acetylated in AWN-1; °, AQN-3 also exists as a glycosylated form; N, is the conserved glycosylation point in PSP-I and the AQN-3 isoform. Two disulphide bridges between nearest-neighbour cysteine residues are conserved within the boar spermadhesins.

The major boar sperm-associated heparin-binding proteins belong to the spermadhesin family

The boar seminal plasma and sperm-associated heparin-binding proteins have been isolated using affinity chromatography on Sepharose-heparin [27]. A minor 18 kDa glycoprotein with an N-terminal amino acid sequence DQHLPGRFLXPAITSDDKCVFPFIYKGNL... was characterized [27]. This clearly showed that boar spermatozoa also possess a heparin-binding protein of the same protein family as the bovine polypeptides. However, the major heparin-binding components, designated AQN-1, AQN-2 (PSP-I), AQN-3, AWN-1, and AWN-2 belong to a novel protein family for which the term "spermadhesin" has been coined (reviewed in [6]). The nomenclature used for boar spermadhesin was based on the first three amino acid residues of their sequence, alanine(A)-glutamine(Q)-asparagine(N) and alanine(A)-tryptophan(W)-asparagine(N); a number indicates the reverse-phase HPLC elution order of polypeptides containing the same N-terminal sequence. These proteins are 111-133 amino acid long, contain two conserved disulphide bridges between nearest-neigbor cysteine residues, and have 40-60% sequence identity (Fig. 2). Posttranslational modifications contribute to the diversity of the family: AWN-2 is identical as AWN-1 but has an acetylated N-terminal alanine residue; PSP-I is constitutively glycosylated; AQN-3 and AWN (isoforms 1 and 2) are found as both non-glycosylated and as N- and O-glycoforms. On the other hand, no glycosylated isoforms of AQN-1 have been reported.

Spermadhesins were first identified by their carbohydrate-binding, and zona pellucida-binding capabilities [6]. Subsequently, it was shown that, in addition to their lectin-like activity, boar spermadhesins are multifunctional proteins which combine within the same molecule heparin- (AQN-1, AQN-2, AQN-3, and AWN) and/or serine proteinase inhibitor- (AQN-1 and AWN) binding abilities. It has been proposed that serine proteinase once bound to sperm surface acceptor molecules, may stabilize or protect sperm surface membrane specific sites for sperm-egg interaction [26]. Inhibitors are then released from the sperm surface during sperm residence in the

female genital tract allowing zona pellucida-binding sites to become exposed. Therefore, the spectrum of ligand-binding strongly suggests that boar spermadhesins may play a role in at least two important aspects of fertilization, sperm capacitation and gamete interaction.

The major biological source of spermadhesins is the secretion of the seminal vesicle epithelium, where the concentration of different spermadhesins ranges from 0.6-7.2 mg/ml [13]. However, AWN-1 is also synthesized by the tubuli recti and the rete testis [31] and is the only spermadhesin found on the surface of epididymal sperm. The amount of coated AWN-1 (5.9-7.5 x 10^6 molecules/epididymal spermatozoon) is sufficient to cover one-third of the entire surface of the sperm head, i.e. the acrosomal cap, with a one-molecule-thick layer. We hypothesize that AWN-1 may be one of the factors contributing to the fertilizing activity of epididymal spermatozoa. Following ejaculation, 12-60 x 10^6 molecules of each AQN-1, AQN-2, AQN-3, and extra 50 x 10^6 AWN molecules (isoforms 1 and 2) become adsorbed on the apical third of the acrosomal cap of spermatozoa, the place where porcine sperm initiate binding to the zona pellucida of the oocyte. However, approximately 60% of adsorbed spermadhesins AQN-1, AQN-2, and AQN-3 are released after 3 h *in vitro* capacitation, the amount of AWN-1 decreases to the level found on epididymal sperm, and the whole AWN-2 population is lost. This figure changes only slightly upon 24 h capacitation.

Spermadhesins AWN-1 and AQN-3, as monomers, possess binding affinity for phosphorylethanolamine (PE) matrices, and the PE-binding site is different from the carbohydrate-recognition domain (unpublished results). Phosphorylethanolamine is a major substituent of boar sperm membrane phospholipids [33]. This suggests that AWN-1 and AQN-3 may bind directly to sperm membrane lipids while the other spermadhesin moieties may coat on top of them as aggregated molecules, and indicates that different subpopulations of spermadhesins may play diverse roles as either decapacitation or acrosome stabilizing factors, positive capacitation elements, and/or receptors for zona pellucida.

Glycosylated isoforms of spermadhesins AQN-3, PSP-I, and AWN bind heparin but fail to bind zona pellucida glycoproteins and soybean trypsin inhibitor [7, 8, 9]. Therefore, attachment of a glycosyl moiety may modulate the receptor function of spermadhesins isoforms, i.e. switching the receptor function between a capacitation factor (heparin-binding) and a primary zona pellucida-binding molecule. The functional inability of glycosylated spermadhesins has been found to be due to steric blockade of the ligand-binding site which results from attachment of a single oligosaccharide to each glycoform, either N-linked to Asn^{50} or O-linked to Ser^{52} or Thr^{95}. This indicates that the zona pellucida- and the inhibitor-binding sites may be located around the glycosylated residues and are different from the heparin-binding site. AWN possesses a consensus sequence for heparin-binding (^{3}NRRSRS^8) which, in a recently developed three-dimensional model for spermadhesins [6], is located at the opposite side of the proposed zona pellucida/inhibitor binding domain. These arrangement of binding domains would be in agreement with the experimental results. Nevertheless, the exact three-dimensional structure and epitope topography of spermadhesins, as well as the way in which the heparin-like glycosaminoglycan- and zona pellucida glycoprotein-binding information is transduced, require further investigation.

Stallion spermatozoa possess heparin-binding proteins of both the BSP and the spermadhesin family

The heparin-binding proteins on ejaculated stallion spermatozoa have recently been isolated and structurally characterized [4]. The major components, termed HSP-1 and HSP-2, belong to the same protein family as bovine BSP- ($A_{1/2}$ and A_3). We estimate that, together, HSP-1 and HSP-2 may account for over 70% of the total sperm-associated heparin-binding proteins.

The primary structure of HSP-1 has been completed [3]. Interestingly, it is a mosaic protein which consists of 121 amino acid residues organized in two types of homologous repeats arranged in the pattern AA'BB' (Fig. 1B). Each of the N-terminal 13-15 residues long A-type

repeat (residues 1-15 and 16-28) contains two O-linked oligosaccharide chains each (threonine residues 5, 12, 22, and 27) and they have 47% sequence identity. The B-type repeats span 44-47 amino acids each (residues 29-72 and 75-121), are not glycosylated, show 43% sequence identity, possess two disulphide bridges (between cysteines, 34-58, 48-72, 80-106, and 94-121) and have the consensus pattern of the fibronectin type-II module. The sequence of the A' domain can only be aligned with the bovine proteins by introducing numerous gaps and the N-terminal A-domain is absent in bovine BSP-$A_{1/2}$ (PDC-109) and BSP-A_3 (Fig. 1A). In addition, only the glycosylation site at threonine 22 is conserved in BSP-$A_{1/2}$. On the other hand, the B-domains are highly conserved in bovine BSP-$A_{1/2}$ and BSP-A_3 proteins (Fig. 1A). However, the critical residues for gelatin-binding are only conserved in the second (B') repeat. Altogether, it seems reasonable to propose that the different combination of structural domains of the bovine and equine BSP-proteins could confer species-specific properties on a common heparin-binding polypeptide framework. Further characterization of the topographic localization, sperm surface acceptor molecules and coating dynamics, may help to clarify this point.

Another abundant sperm-associated heparin-binding protein is a 16 kDa component which may represent some 20% of the total stallion sperm-associated heparin-binding proteins. This 16 kDa protein is not glycosylated and is immunologically related to boar AWN spermadhesin [4]. In addition, initial structural characterization of this stallion AWN-protein shows that it shares a high degree of amino acid sequence with the boar protein (unpublished results). However, whereas the boar AWN spermadhesin binds homogeneously to the whole acrosomal cap surface [28], an indirect immunofluorescence study showed that the topographical localization of the 16 kDa AWN-related protein is restricted to the equatorial segment in specimens of both epididymal and ejaculated stallion spermatozoa [4]. AWN is the only member of the equine spermadhesin family found on either seminal plasma or spermatozoa.

In addition to proteins of the BSP and spermadhesin families, a minor component (25 kDa), which probably represents less then 10% of the total heparin-binding proteins, with the N-terminal amino acid sequence IIGGWEXEKHSKPWQVAVYHQGHFQXG..., has also found associated with ejaculated stallion spermatozoa. This sequence shows a high degree of sequence identity with serine proteinases of the kallikrein (EC 3.4.21.35) family, in particular with human prostate-specific antigen, PSA (27 kDa) [20], and with the androgen-dependent arginine-esterase (EC 3.4.21.34) mRNA product of canine prostate [11]. Human PSA is one of the enzymes involved in cleaving structural proteins of the seminal coagulum [19]. Its function in stallion sperm physiology has not been assessed, and deserves further study.

Heparin-binding proteins on human sperm: kallikrein and spermadhesins

Recently our laboratories have begun to characterize the human seminal plasma heparin-binding proteins which bind to the sperm surface. Using affinity chromatography, two major components (80 kDa and 28 kDa) have been isolated. Each protein accounts for around 50% of the total heparin-binding proteins in seminal plasma. The 28 kDa protein contains an amino acid sequence identical with previously characterized human plasma (glandular) kallikrein. The structure of the 80 kDa component(s) remains to be established. In addition, using indirect immunofluorescence microscopy, we have localized the binding site of an AWN-crossimmunoreacting protein to the equatorial segment of ejaculated human spermatozoa. Its relative abundance on spermatozoa has not been studied, however. Although the biological significance of these human molecules on sperm function remains obscure, we are currently elucidating the primary structure of human AWN as a first step towards determining its function.

Conclusion

The mechanism by which heparin modulates capacitation and/or the acrosome reaction is poorly understood. A major event in capacitation is believed to be the removal or alteration of a

protective coat from the sperm plasma membrane. This structural alteration may correlate with loss or reduction of plasma membrane cholesterol, uptake of extracellular calcium, and elevation of the internal pH, all of which constitute important steps in the capacitation and early steps of the acrosome reaction. The biological effects of heparin is not absolutely conserved in all mammalian species. Since, in bull, boar, stallion, and man, the seminal plasma heparin-binding proteins which become bound to the sperm surface belong to only three different protein families (Table 1), our working hypothesis is that species-specific modulation of sperm capacitation/acrosome reaction exerted by heparin might be related to a combination of , at least, the following factors:

The chemical structure, amino acid sequence and posttranslational modifications, of the heparin-binding proteins bound to the sperm surface;

The relative abundance of members of the different heparin-binding protein families in the seminal plasma;

The topography and amount of the different heparin-binding proteins on the surface of spermatozoa during capacitation.

TABLE 1. — Relative distribution of heparin-binding proteins of different protein families on mammalian spermatozoa. NF, not found.

Species	Protein family		
	BSP	Spermadhesin	Kallikrein
Bovine	++++	NF	NF
Pig	+	++++	NF
Horse	+++	++	+
Human	NF	++	++

To dissect the relative biological relevance and synergy of these, and possibly other, factors in heparin-mediated sperm capacitation is one of the main goals of our laboratories.

ACKNOWLEDGEMENTS

The work from the authors' laboratories has been financed by grants Tö 114/3-1 (E.T-P.) from the Deutsche Forschungsgemeinsam, 01KY9103 from Bundesministerium für Forschung und Technologie (E.T-P.), Germany, and PB92-0096 from the Dirección General de Investigacion Científica y Técnica (J.J.C., L.S.), Spain.

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